Patulin

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The capacity of various fungi to produce toxic metabolites is well established, but the potential role that many of these toxic compounds play in disease processes is not as well understood and is, more often than not, uncertain at best. Hence, for obvious reasons, it would appear desirable to develop a data bank on these compounds. One of the metabolites that falls into this uncertain category with respect to involvement in mycotoxicoses is patulin, an antibiotic furopyrone, empirical formula $C_7H_6O_4$, molecular weight 154; colorless to white crystals melting at 110.5, optically inactive with a single peak in the ultraviolet at 276 nm. It is a neutral substance which is soluble in water and most organic solvents except pentane-hexane. Patulin's chemical structure was determined by Birkinshaw *et al.* (1943) (Fig. 1) when interest was high in its antibiotic properties.

Detailed reviews on patulin covering almost all aspects, chemical and biological, have been published over a period of years (Florey *et al.*, 1949; Singh, 1967; Korzybski *et al.*, 1967; Ciegler *et al.*, 1971; Scott, 1974; Stott and Bullerman, 1975; Wilson, 1976). However, since the objective of this conference is an attempt to define the actual risks of mycotoxins in human and animal health, this review will confine itself to those aspects.

A number of fungi comprising several species and genera are capable of producing patulin, which precludes use of this compound as a biochemical taxonomic feature. Curiously, most of the known producers were detected and identified in the 1940's with only the unusual species, *Penicillium lapidosum*, being added to the list in 1967 (Table 1). This may have occurred as a result of the intense search for antibiotics conducted during that period. A perusal of the species listed in Table 1 indicates that from the practical standpoint of potential contamination of food and feedstuffs, only the following organisms might be of concern: *Penicillium urticae, P. expansum, P. melinii, P. cyclopium, Aspergillus clavatus, A. terreus, Byssochlamys nivea.* Again, one should carefully distinguish between the ability of cultures to produce

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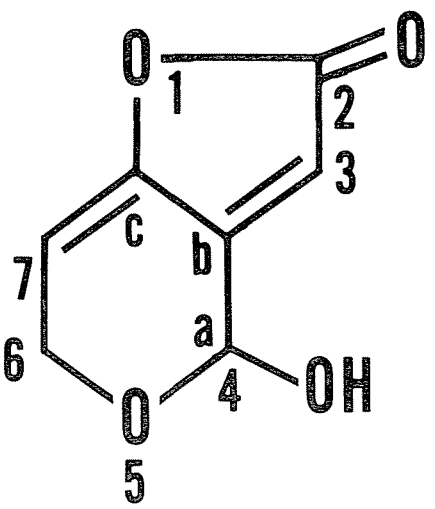


Figure: 1. Chemical structure of patulin.

meaningful quantities of toxin under laboratory conditions and what may actually occur in nature. Contamination of foods and feeds with patulin-producing molds does not necessarily mean that toxin formation automatically ensues, particularly since mixtures of competing cultures usually occur rather than the pure culture situation that exists in the laboratory. Table 2 lists those commodities reported to be contaminated with fungi capable of patulin production. The ability to produce patulin has been tested on a number of commodity substrates other than mycological media (Table 3). However, the proven natural contamination of patulin on agricultural products has been limited to apples and apple cider or apple juice (Brian *et al.*, 1956; Scott *et al.*, 1972; Harwig *et al.*, 1973a,b; Drilleau and Bohuon, 1973; Wilson and Nuovo, 1973; Ware *et al.*, 1974; Eyrich, 1975). Levels of toxin found ranged from 45 ppm in cider (Wilson and Nuovo, 1973) to 1,000 ppm in apple sap (Brian *et al.*, 1956). The implication of these findings to human health cannot as yet be evaluated.

Data implicating patulin in mycotoxicoses are primarily circumstantial and involve cattle in all cases. Japanese investigators isolated *P. urticae* from a malt feed believed responsible for the deaths of over 100 dairy cattle in Japan (Ukai *et al.*, 1954; Hori and Yamamoto, 1953; Yamamoto, 1954a,b). They subsequently fed malt grain inoculated with the isolated culture to a bull as well as to mice; test results included nervous symptoms, brain hemorrhage,

Table 1: Patulin-producing fungi

Fungal species	Synonym	Reference
Penicillium urticae Bainier	*Penicillium griseo-fulvum* Dierckx	Ken and Heatley 1945
	Penicillium patulum Bainier	*Chain et al.* 1942 Birkinshaw *et al.* 1943
Penicillium expansum Link	*Penicillium leucopus* (Pers.) Biourge	Anslow *et al.* 1943
Penicillium cyclopium Westling		Efimenko and Yakimov 1960
Penicillium granulatum Bainier	*Penicillium divergens* Bainier and Sartory	Barta and Mecir 1948
Penicillium claviforme Bainier		Bergel *et al.* 1943
Penicillium melinii Thom		Karow and Foster 1944
Penicillium novae-zeelandiae van Beyma		*Burton and Pausacker 1947*
Penicillium lapidosum Raper and Fennell		Myrchink 1967
Penicillium terrestre Jensen	*Penicillium equinum* van Beyma	Burton and Pausacker 1947
Aspergillus clavatus Desm.		Umezawa *et al. 1947*
Aspergillus giganteus Wehmer		Florey *et al.* 1944
Aspergillus terreus Thom		Kent and Heatley 1945
Byssochlamys nivea Westling	*Gymnoascus* sp.	Karow and Foster 1944

and death (Hori *et al.*, 1954). In a similar study, Capitaine and Balouet (1974) injected patulin ip into mice and induced symptoms similar to those observed in cattle which had consumed forage infected by *P. urticae*. Histopathological observation showed congestive lesions in miscellaneous organs and neural degeneration at the level of the cerebral cortex. However, there appears to be no direct action of patulin on the brain. In France, pulmonary edema and congestion were noted in dead cows which had been fed wheat contaminated with *A. clavatus* (Moreau and Moreau, 1960; Jacquet *et al.*, 1963). The same mold species was found in malt that had intoxicated cattle in Germany (Schultz, 1968; Schultz *et al.*, 1969). However, in another study, Ohkubo *et al.* (1955) stated that they could not find *P. urticae* in malt rootlets suspected to be involved in poisoning of dairy cattle. Extensive laboratory studies on farm animals have not been reported and would obviously be desirable.

Toxicological studies to date have been confined to laboratory animals. The LD_{50} in a variety of biological systems is shown in Table 4. In rodents death is often accompanied by convulsions and edematous and hemorrhagic lungs, as well as edema of the subcutaneous tissues; in rats almost no urine is voided up until death (Katzman *et al.*, 1944). Toxic effects in other biological systems have recently been reviewed (Stott and Bullerman, 1975).

Of equal importance, however, is the report of Dickens and Jones (1961) that patulin, upon subcutaneous (sc) injection of 0.2 mg patulin in arachis oil twice a week for 61 to 64 weeks, resulted in sarcomas in six of eight male rats; tumors were confined to the site of injection. Patulin has not been shown

Table 2: Commodities contaminated with patulin-producing fungi

Commodity	Fungi	Reference
Wheat flour	*Aspergillus terreus* *Aspergillus clavatus* *Penicillium urticae*	Graves and Hesseltine 1966 Bullerman and Hartung 1973
Refrigerated dough products	*Aspergillus terreus* *Penicillium urticae*	Graves and Hesseltine 1966
Cereals, legumes	*Penicillium expansum* *Penicillium urticae* *Aspergillus clavatus* *Aspergillus terreus* *Byssochlamys nivea*	Scott 1964 Scurti *et al.* 1973
Pecans	*Penicillium expansum*	Schindler *et al.* 1974
Fruits		
Apricots, crab apples, persimmons, pears, grapes, apples	*Penicillium expansum* *Byssochlamys nivea*	Sommer *et al.* 1974, Yates 1974, Anslow *et al.* 1943, Harwig *et al.* 1973a, Wilson and Nuovo 1973, Atkinson and Stanley 1943
Fruit juices	*Byssochlamys nivea*	Yates 1974
Meat	*Penicillium expansum*	Ciegler *et al.* 1972
	Penicillium urticae	Tauchman *et al.* 1971
	Penicillium melinii	Wu *et al.* 1974, Fiedler 1974
	Penicillium claviforme	Engel *et al.* 1975
Poultry feed		Lovett 1968, Ciegler *et al.* 1972
Cheese		Bullerman and Olivigni 1974, Bullerman 1976
Bread		Bullerman and Hartung 1973

Table 3: Patulin-production on agricultural commodities

Commodity	Fungi	Reference
Fruits		
Apples	*Penicillium expansum*	Brian *et al.* 1956 Wilson and Nuovo 1973 Sommer *et al.* 1974 Lovett *et al.* 1975
Stone fruits	*Penicillium expansum* *Aspergillus clavatus* *Penicillium claviforme* *Penicillium urticae*	Buchanan *et al.* 1974 Lovett *et al.* 1974
Meat	*Penicillium expansum*	Alperden *et al.* 1973
Straw	*Penicillium urticae* *Byssochlamys nivea*	Norstadt and McCalla 1971 Escoula 1975
Bread	*Penicillium expansum*	Reiss 1973

Table 4: Patulin LD_{50} in biological systems

Test system	LD_{50} mg/kg[a]	Reference
Mouse	8-10 sc	Katzman *et al.* 1944
	15 sc	Broom *et al.* 1944
	15.6 iv	Yamamoto 1954
	25 iv	Broom *et al.* 1944
	5.7 ip	Ciegler *et al.* 1976
	15 ip	Hofmann *et al.* 1971
	15 ip	Broom *et al.* 1944
	30 ip	Andraud *et al.* 1964
Rat	15 sc	Broom *et al.* 1944
	25 sc	Katzman *et al.* 1944
	25.50 iv	Broom *et al.* 1944
Chick	170 po	Lovett 1972
Chick embryo (4-day-old)	2.35 μg/embryo	Ciegler *et al.* 1976
Chang liver cells	1.85 μg/ml	Schaeffer *et al.* 1975
Zebra fish larvae	18.0 μg/ml	Abedi and Scott 1969

to be a carcinogen by per os (po) administration, and the validity of sc injection as a means of testing for carcinogenicity should be questioned.

The early findings that patulin had antibiotic activity led to its testing against the common cold in humans. Initial favorable reports (Gye, 1943; Hopkins, 1943) could not be later confirmed (Medical Research Council, 1944; Stansfeld *et al.*, 1944). Topical application led to dermal irritation in humans (Dalton, 1952) and animals (Hofmann *et al.*, 1971) as well as to stomach irritation, nausea, and vomiting in humans when given orally (de Rosnay *et al.*, 1952; Freerksen and Bönicke, 1951; Walker and Wiesner, 1944). In contrast, intravenous (iv) perfusion of 0.1 grams into a human had no ill effects (de Rosnay *et al.*, 1952). These data would tend to cast some doubt on the seriousness of patulin as a mycotoxin in humans. However, there is always the possibility of cumulative toxic action, although the evidence for this type of effect by patulin is contradictory. Broom and his colleagues (1944) dosed mice ip daily for 2 weeks with 0.5 mg toxin/20 grams and killed about 50% of the animals. In chronic experiments, microscopic lesions resulted at the site of sc injection, but only a slight loss of weight resulted on ip administration. Cumulative toxicity in mice was noted by de Rosnay *et al.* (1952) and Lembke and Hahn (1954) after daily sc and ip injections of 0.1 mg for up to 4 weeks; liver lesions occurred in chicks dosed daily po with 0.2 mg for 6 weeks. In contrast, Freerksen and Bönicke (1951) and Lembke and Hahn (1954) noted no effects in mice on cumulative po dosing. Clearly, additional toxicological research is needed.

On a molecular level, patulin appears to inhibit aerobic respiration (Singh, 1967), effect some aspects of membrane permeability (Kahn, 1957), reduce adenosine triphosphatase activity (Andraud and Andraud, 1971), cause DNA-strand breakage of Hela cells (Umeda *et al.*, 1972) and chromosomal aberrations (Withers, 1966; Reiss, 1975); protein, DNA, and RNA syn-

thesis do not appear to be affected (Rubin and Giarman, 1947; Singh, 1967).

Another aspect of the problem that should be considered with respect to potential toxicity is the stability of patulin in the various commodities and environments in which it might occur. This problem is more complex than is at first apparent and has interesting ramifications.

The stability of patulin in fruit juices, wet and dry corn, wheat and sorghum, and cheese has been investigated; it is stable in grape and apple juice and dry corn, but not in orange juice, flour, baked bread, cheese, wet corn, or apple juice fermented with *Saccharomyces* spp. (Stott and Bullerman, 1976; Harwig *et al.,* 1973a,b; Pohland and Allen, 1970; Scott *et al.,* 1972; Scott and Somers, 1968). Disappearance of patulin in these various commodities has been attributed to its ready reaction with sulfhydryl-containing amino acids or proteins, leading some workers to assume that patulin is inactivated or destroyed in the process (Ashoor and Chu, 1973a,b; Atkinson and Stanley, 1943; Dickens and Cooke, 1965; Geiger and Conn, 1945; Hofmann *et al.,* 1971). However, a careful perusal of the limited available published data pertaining to biological activity indicates that this assumption may be only partially correct. Patulin-cysteine adducts were still partially bacteriostatic to selected Gram + and Gram - bacteria (Geiger and Conn, 1945), and they were still capable of partial inhibition of rabbit muscle aldolase, a thiol-containing enzyme (Ashoor and Chu, 1973b). The mechanism of the reaction is complex and can be postulated to involve a Michael addition[1] of the sulfhydryl group to the double bond of the unsaturated lactone system of patulin. However, the products of a reaction between patulin and various sulfhydryl-containing substances have never been characterized. We are currently studying the mechanism of the reaction between patulin and various thiol and sulfhydryl-containing compounds such as cysteine and glutathione. In buffer, the reaction proceeds rapidly with formation of acid. In unbuffered solutions, about 1 mole of base per mole of patulin is needed to maintain a constant pH of 5.4. When 2 moles of cysteine per mole of patulin are present, the final amount of base required to maintain a constant pH corresponds to the average molar concentration of cysteine plus patulin, which suggests that one molecule of patulin can react with more than one molecule of cysteine. In buffered solutions, the reaction proceeds very slowly. Thin-layer chromatography shows the presence of at least four ninhydrin-positive and two ninhydrin-negative compounds. We are attempting to characterize these substances.

Hofmann and his colleagues (1971) had postulated a reaction mechanism involving addition of thiol at positions 4 and 7. However, an examination of the molecule indicates the possibility of a variety of resonance forms with at least six sites (2, 3, 4, b, c, 7) capable of undergoing nucleophilic attack (Fig. 2). Patulin has a chiral center at position 4; position 7 can also become

[1] Nucleophilic addition of a carbanion to an α,β-unsaturated carbonyl compound.

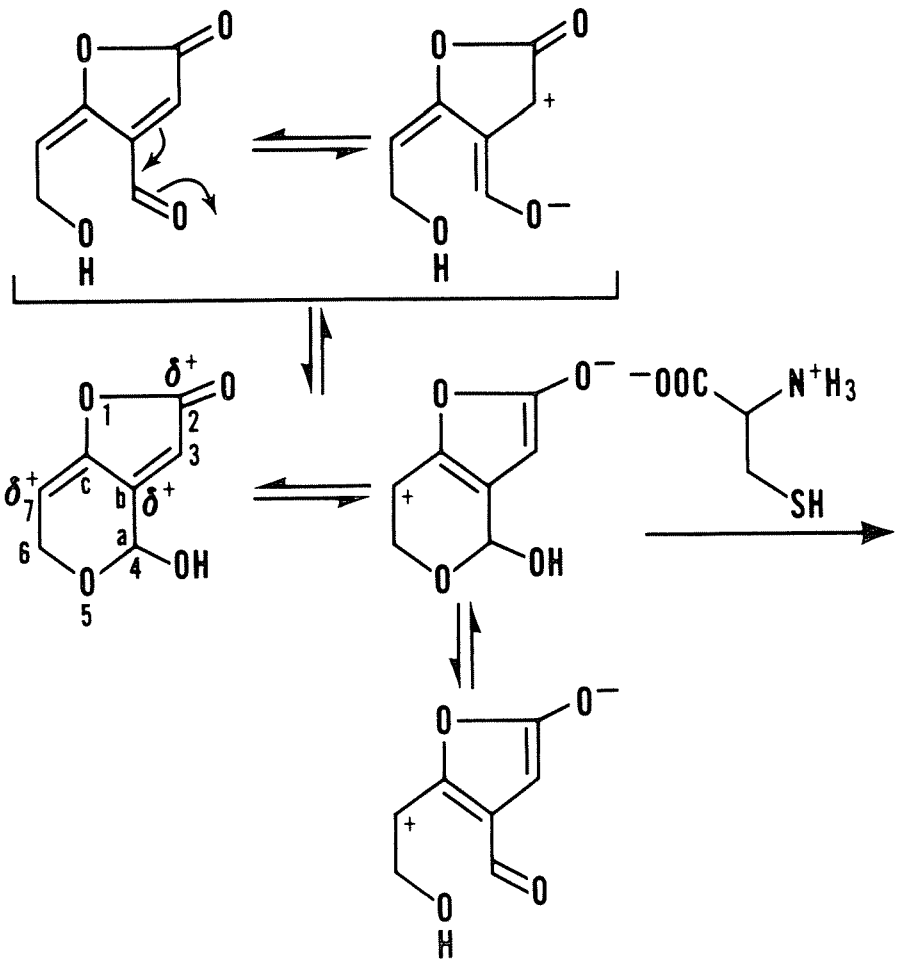
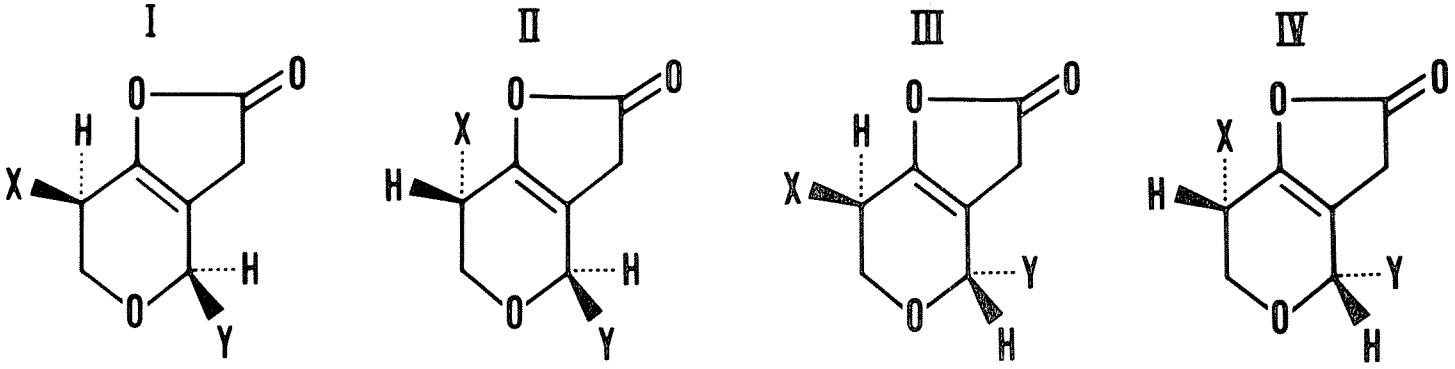


Figure: 2. Possible resonance forms of patulin.

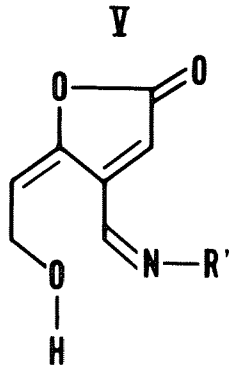
a chiral center upon an addition reaction at that site permitting formation of further potential isomers (Fig. 3a, 3b). Also, cysteine is a trifunctional compound; attack by the thiol group need not be the initial reaction, although a study by Friedman *et al.* (1965) predicts, based on kinetic data, that α,β-unsaturated compounds should react preferentially with sulfhydryl groups. That both the sulfhydryl and amino group of cysteine may react with patulin is indicated by the data of Ashoor and Chu (1973a,b) and by our own study in which at least two ninhydrin-negative compounds are formed. The four ninhydrin-positive products detected could involve Michael addition of the nucleophilic thiol group in cysteine to the conjugated double bonds of patulin. The cysteine moiety can then theoretically cyclize, leading to further reaction products (Fig. 4); these reactions can account for the acid generated.

We have tested the patulin-cysteine reaction mixture for toxicity to the mouse and chick embryo. In the mouse, ip injection of four times the patulin

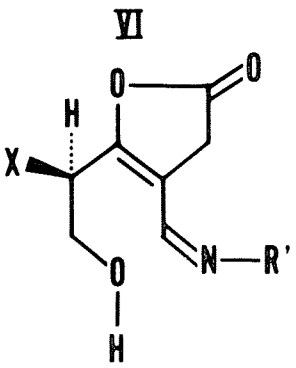


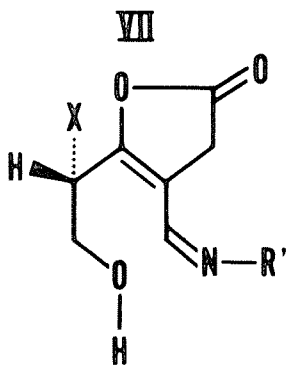
				Ninhydrin
I	X = R—S	Y = OH	4 isomers	+
II	X = R—S	Y = R—S	4 isomers	+
III	X = R'—NH	Y = OH	4 isomers	—
IV	X = R'—NH	Y = R—S	4 isomers	+

Figure: 3. Potential adducts formed by reaction of patulin with sulfhydryl compounds.

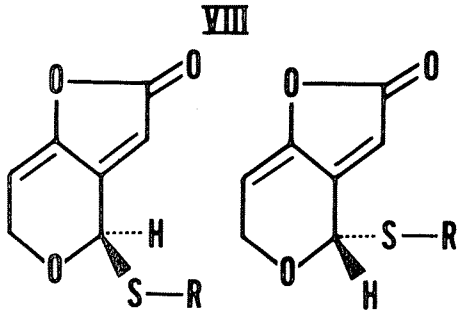


Ninhydrin –





		Ninhydrin
VI	X = R—S	2 isomers +
VII	X = R'—NH	2 isomers –



Ninhydrin +
2 isomers

8 Chemical compounds and isomers

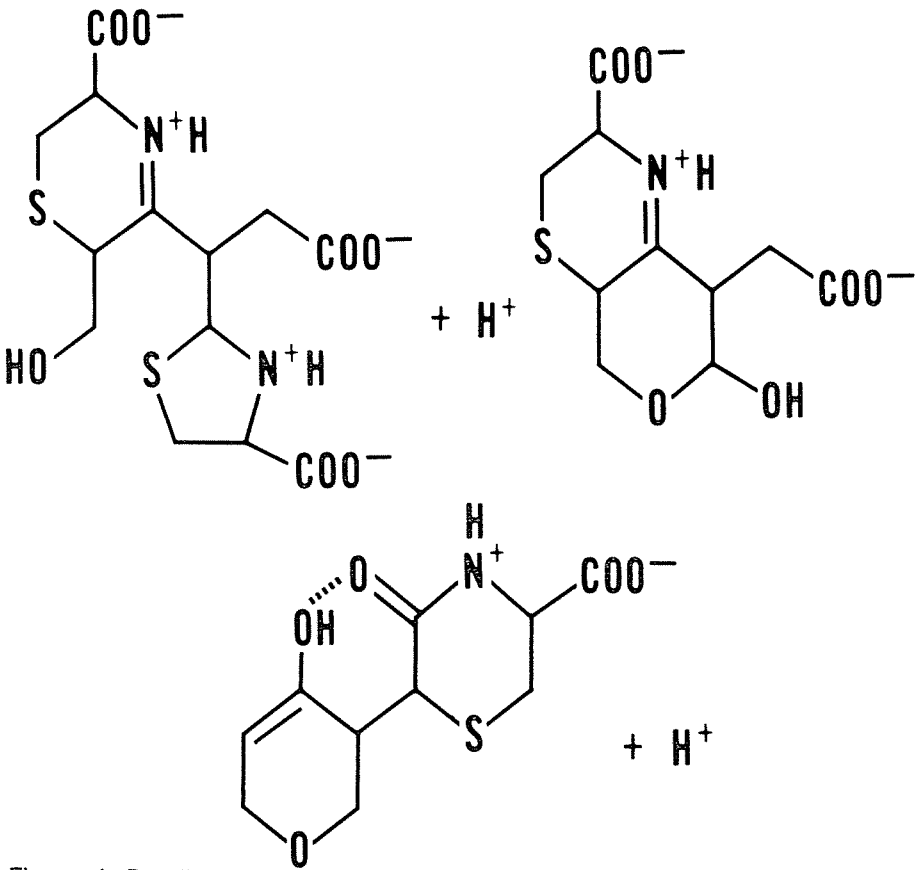


Figure: 4. Possible heterocyclic structures produced from reaction of patulin with cysteine.

equivalent of the LD_{50} dose had no discernible toxic effects. In the chick embryo, no lethality was observed at dosages of about 50 times the calculated LD_{50} of patulin equivalents (Ciegler *et al.*, 1976). However, the reaction mixture retained some terotogenicity to the 4-day-old chick embryo (Table 5). The possibility of teratogenicity in mammalian systems has not been investigated.

Most research involving mycotoxins has been carried out using pure compounds under well-controlled conditions. It is obvious that such ideal situations do not exist in mycotoxicoses in nature. In fact, mycotoxicoses probably represent a more complex manifestation of interactions between susceptible hosts and etiological agents than was initially surmised. In addition to the physiological variables inherent to the host, more than a single toxin may be involved, which may result in potential synergistic activity. We recently reviewed this complex subject in depth (Lillehoj and Ciegler, 1975), and I will not go into the details here except as it pertains to patulin. Harwig *et al.* (1973) in Canada had isolated strains of *P. expansum* from natural rots of

Table 5: Response of the chick embryo to patulin and patulin-cysteine adducts (Ciegler et al. 1976)

Dose/egg μg	Number survivors/25 eggs at 20 days	Survivors showing teratogenic effects	% Teratogenic
Patulin			
1	17	8[a]	47
2	14	5[a]	36
4	8[b]	0	—
6	2[b]	0	—
Patulin-cysteine adduct			
3	25	0	0
15	24	3[a]	12
75	23	3[a]	13
150	25	5[a]	20
Fertility control	24	0	0
Drilled-only control	25	0	0
Solvent control	24	0	0

[a] Embryos were small (average weight ratio to controls of 1:1.9) and showed primarily ankle malrotation and splayed feet. One embryo each at the 1 and 2 mg patulin dose levels exhibited exencephaly, exophthalmia, and crossed malformed beaks.
[b] Surviving embryos were about one-half the weight and size of control embryos.

apples that were capable of synthesizing both patulin and citrinin. During an analysis of the mycotoxin-producing potential of molds isolated from mold-fermented sausage in Germany (Ciegler *et al.*, 1972), we isolated three strains of *P. expansum* also capable of producing both patulin and citrinin. Two of the cultures were unstable, one rapidly losing the ability to produce both toxins and the second the ability to produce patulin. The rapid loss of toxin-synthesizing ability of fungi isolated from natural sources onto mycological media is a subject worthy of more research.

Data obtained from simultaneous administration to 4-day-old chick embryos of varying ratios of patulin plus citrinin was plotted as an isobologram according to the method of Hewlett (1969). The straight dashed line represents the expected LD_{50} of toxin combinations based on a simple additive response. Data from two experiments fall along this line indicating an additive effect from patulin plus citrinin dosing (Fig. 5).

In summary, patulin is a relatively toxic secondary metabolite whose exact role in mycotoxicoses is yet to be delineated. It can be produced by a variety of molds but, from a practical standpoint, *P. expansum* occurring on apples and apple cider appears to be the most important; only this fruit has been found to be contaminated with patulin. Patulin-producing fungi have been found on other food and feed commodities but the toxin itself has not been detected.

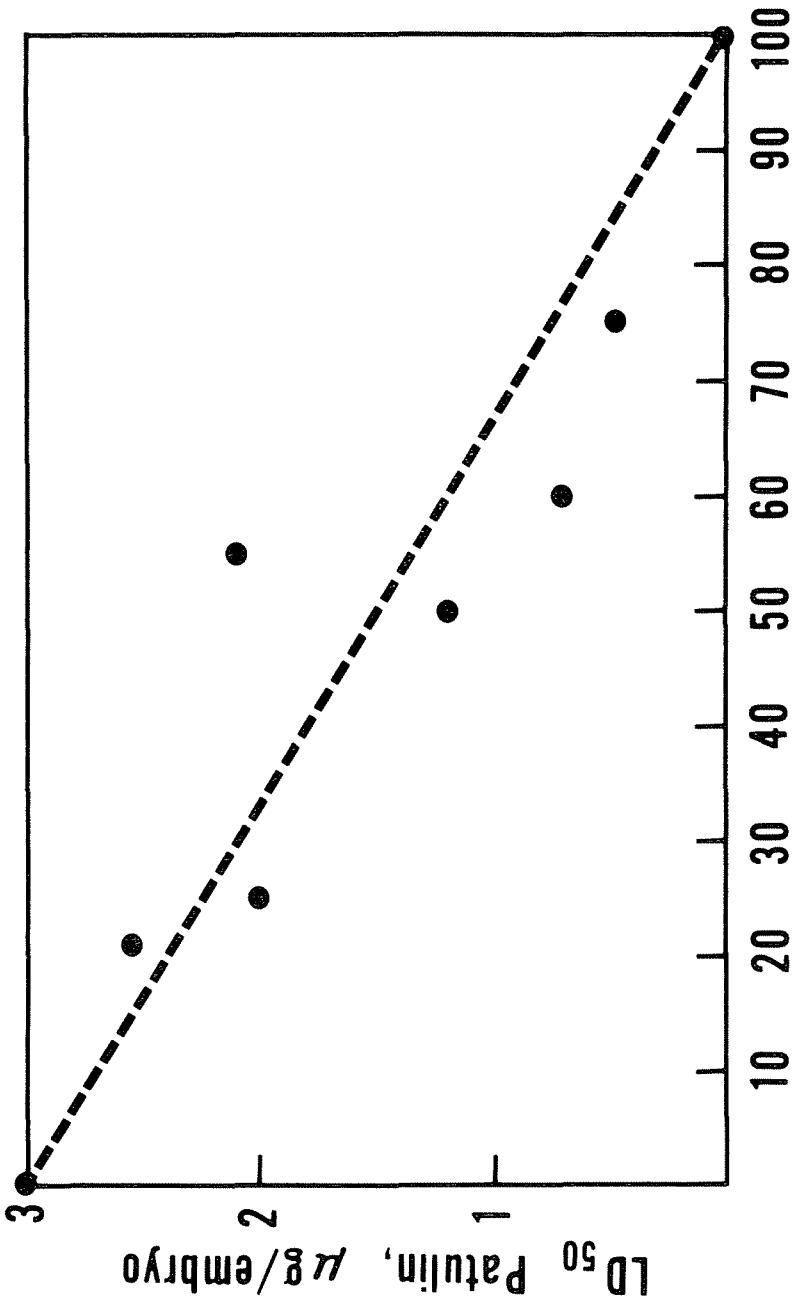


Fig. 5. Isobologram of LD_{50}'s in 4-day-old chick embryos dosed with various ratios of patulin plus citrinin.

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